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Original Article

Cytochemical Changes in Hepatocytes of Rats With Endotoxemia or Sepsis: Localization of Fibronectin, Calcium, and Enzymes

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Bacterial lipopolysaccharide (LPS) is known to be implicated in the pathogenesis of endotoxemia and septic shock. The liver is the first vital organ to exhibit pathological alterations in shock. The present studies include immunoelectron microscopic localization of tissue fibronectin and cytochemical localization of calcium and enzymes in hepatocytes of animals with LPS-induced endotoxemia or cecal ligation-induced septic shock. The results showed increased staining of fibronectin in the basal (perisinusoidal) surfaces and in the cisternae of rough endoplasmic reticulum and the Golgi complex of hepatocytes in rats with endotoxemia or septic shock. Intracellular calcium content was significantly increased in the LPS-treated or septic rats. Calcium pyroantimonate precipitate was deposited predominantly on the outer surfaces of the RER of hepatocytes. In addition, diminution or depletion of glycogen, reduction of catalase-containing peroxisomes, increase of G-6-Pase activity, and depletion of cytochrome *c* oxidase in many mitochondria were also observed in hepatocytes of experimental animals. The overall results suggest that LPS stimulates: (a) hepatic synthesis and secretion of fibronectin; (b) uptake of calcium by hepatocytes; and (c) G-6-Pase activity. LPS treatment also leads to reduced numbers of peroxisomes and depletion of cytochrome *c* oxidase. (*J Histochem Cytochem 36:665–678, 1988*)

KEY WORDS: Hepatocytes; Immunoelectron microscopy; Ultracytochemistry; LPS; Fibronectin; Calcium; Enzymes

Introduction

Lipopolysaccharides (LPS), gram-negative bacterial endotoxins, are known to be implicated in the pathogenesis of endotoxemia and septic shock (Bradley, 1979; Berry, 1977; Hinshaw, 1976; Nowotny, 1969). The liver, which contains an important component of the reticuloendothelial system involved in host resistance to LPS-related septic shock (Zahlten et al., 1981; Fine et al., 1959; Zweifach, 1958), has been reported to be the first vital organ displaying pathological alterations in shock. Intravenously injected LPS is cleared rapidly from the blood by Kupffer cells in vivo (Maier et al., 1981; Reske et al., 1981; Mathison and Ulevitch, 1979). Impairment of LPS detoxification by Kupffer cells (Cook et al., 1985; Gut et al., 1984; Tanikawa and Iwasaki, 1984; Palmeria and Fine, 1969) often augments the toxicity of LPS and causes hepatic injuries. The activities of some hepatic enzymes, including carbohydrate metabolic enzymes (McCallum, 1981), drug metabolizing enzymes (Yoshida et al., 1982; Egawa and Kasai, 1979), and mitochondrial enzymes (McGivney and Bradley, 1979), are reported to be depressed by LPS. LPS has also been shown to decrease intracellular calcium in certain cells (Kilpatrick-Smith and Erecinska, 1983) and to inhibit synthesis of hepatic enzymes, such as phosphoenolypyruvate carboxykinase (Berry and Rippe, 1973).

In recent years, there has been increased interest in the relationship between fibronectin and sepsis (Velky et al., 1984; Lanser and Saba, 1983; Richards et al., 1983; McCafferty and Saba, 1982; Saba and Jaffe, 1980; Saba and Cho, 1979). Fibronectin comprises a family of high molecular weight glycoproteins consisting of soluble and insoluble forms. Insoluble (tissue) fibronectin is localized on cell surfaces and intercellular matrices; it participates in cell adhesion and also, in part, modulates microvascular integrity, vascular permeability, and wound repair. Deficiency or destruction of tissue fibronectin results in widening intercellular junctions and increased vascular permeability. Soluble (plasma) fibronectin is a nonspecific opsonin which mediates reticuloendothelial clearance of blood-borne particulates, to prevent pulmonary and peripheral vascular embolization and organ injury. Depletion of plasma fibronectin results in depression of reticuloendothelial system phagocytic function. This may potentiate microvascular embolization and sludge in critical illness (Saba and Jaffe, 1980; Scovill et al., 1979; Mosher and Williams, 1978). A recent report suggests that LPS may stimulate synthesis of fibronectin (Velky et al., 1984).

Although the effects of endotoxins on the ultrastructure of liver have been extensively studied (White et al., 1973; Rangel et al.,

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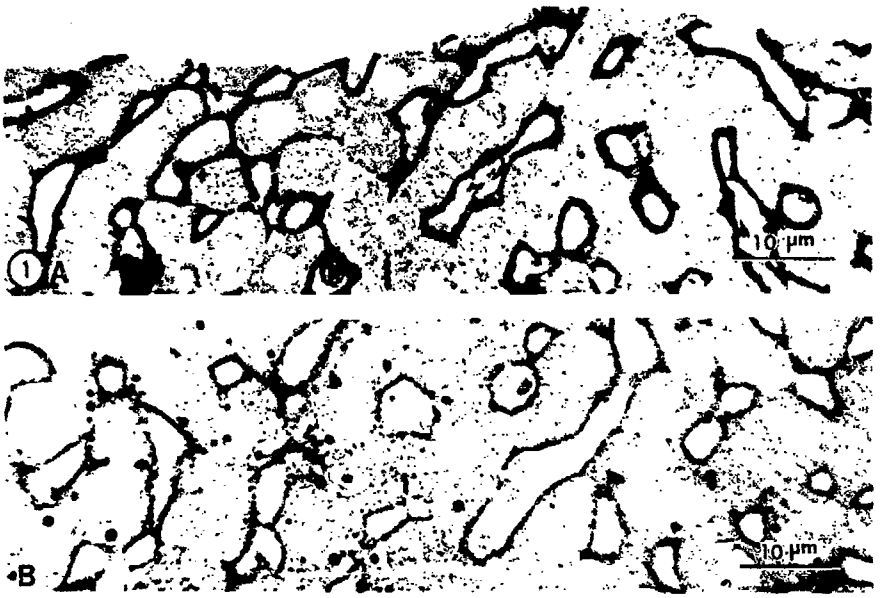


Figure 1. Immunohistochemical localization of tissue fibronectin in rat liver. As indicated by the reaction product of HRP, fibronectin is confined to the periluminal surfaces of sinusoids. The livers of LPS-treated or septic rats (A) show more intense staining for fibronectin than those of the control animals (B). Original magnifications × 1875.

1970; Levy et al., 1968; Boler and Bibighaus, 1967; De Palma et al., 1967), the effects of LPS on fibronectin, calcium, and enzymes in liver has not been demonstrated. In this report, we describe the ultracytochemical localization of tissue fibronectin, calcium, and enzymes in the hepatocytes of rats with LPS-induced endotoxemia or cecal ligation- and puncture-induced septic shock. In addition, the effect of LPS on the permeability and pinocytic activity of liver was also studied, using horseradish peroxidase as a tracer.

Materials and Methods

Animals. Male Sprague–Dawley rats (Charles River; N. Wilmington, MA) weighing 250–350 g were used in the studies. The animals were maintained in standard wire cages in an environmentally controlled room and were provided with food and water ad libitum.

Induction of Endotoxemia and Sepsis. In each experiment, five animals were injected with 10 mg/kg *E. coli* (0111:B4) LPS (Calbiochem; San Diego, CA) in saline via a penile vein; five controls were given saline only. Twenty-four hr after treatment, only animals displaying endotoxemic symptoms, such as piloerection, hypothermia, and diarrhea (Wichterman et al., 1980), were used for studies.

Sepsis was induced by cecal ligation and puncture, as described by Wichterman et al. (1980), with an additional ligation of the cecal ileocolic vascular bundle. Controls were sham-operated. Animals exhibiting "late septic" symptoms, as described by Wichterman et al. (1980), 24 hr after surgery were sacrificed for the studies. Three septic animals and three sham-controls were utilized in fibronectin localization and each enzyme study. All injections and operations were performed under halothane anesthesia.

Fixation. For localization of fibronectin, animals were perfused via the left ventricle for 20 min with 2% paraformaldehyde containing periodate and lysine (McLean and Nakane, 1974). The middle lobes of the liver were excised immediately after perfusion and re-fixed in the same fixative by immersion at 4°C for an additional 20–40 min. Animals were also perfused with 2% glutaraldehyde–1% paraformaldehyde in the same manner for 20 min, for enzyme localization. The middle lobes of the liver were used for enzyme studies. All samples were thoroughly washed in 0.1 M sodium cacodylate buffer (pH 7.2) for at least 24 hr before further processing.

Samples for fibronectin and enzyme localization were sectioned at 70–80 μm using a vibratome (Lancer; Brunswick Co, St. Louis, MO).

Immunocytochemical Localization of Fibronectin. Vibratome (tissue) sections were incubated for 24–48 hr at 4°C in Tris–saline (pH 7.6, with 0.5% saponin) containing a 1:20 or 1:50 dilution of anti-fibronectin antibody conjugated with horseradish peroxidase (HRP) (Accurate Chemical and Scientific Corp; Westbury, NY). Samples were re-fixed in 2% glutaraldehyde–1% paraformaldehyde for 15 min after incubation. After thorough washing in 0.1 M sodium cacodylate buffer, the samples were incubated in 0.05 M Tris buffer (pH 7.6) containing diaminobenzidine (DAB) (Sigma Chemical; St. Louis, MO) and H_2O_2 (Graham and Karnovsky, 1966).

Calcium Localization. Three animals displaying endotoxemic or septic symptoms 24 hr after LPS treatment or surgery, and three control or sham-operated animals, were sacrificed by guillotine. The middle lobes of the livers were excised and promptly immersed in 1% osmium tetroxide containing 2.5% potassium pyroantimonate (pH 7.6) at 4°C for 4–5 hr. Samples were also incubated for 45 min in 1% osmium tetroxide containing 10 mM EGTA (ethylene glycol tetraacetic acid) (Sigma) and then fixed in potassium pyroantimonate as above (Appleton and Morris, 1979). In addition, livers from animals treated with 10 mg/kg LPS for 30 min were also used for calcium localization.

Glycogen. Liver fixed by 2% glutaraldehyde–1% paraformaldehyde was post-fixed overnight in 2% osmium tetroxide containing 1.5% potassium ferrocyanide at 4°C (Fawcett and Dyme, 1974). Paraffin-embedded samples were also used for study of glycogen distribution, using a periodic acid–Schiff (PAS) technique. A diastase digestion method was utilized to selectively eliminate PAS-positive staining attributable to glycogen.

Glucose-6-Phosphatase (G-6-Pase). Vibratome sections from perfused livers were processed for G-6-Pase localization according to the method described by Nicholas et al. (1984).

Cytochrome c Oxidase. Vibratome sections of hepatic tissues from endotoxemic or septic rats were incubated in 2.5 mM DAB in phosphate buffer.

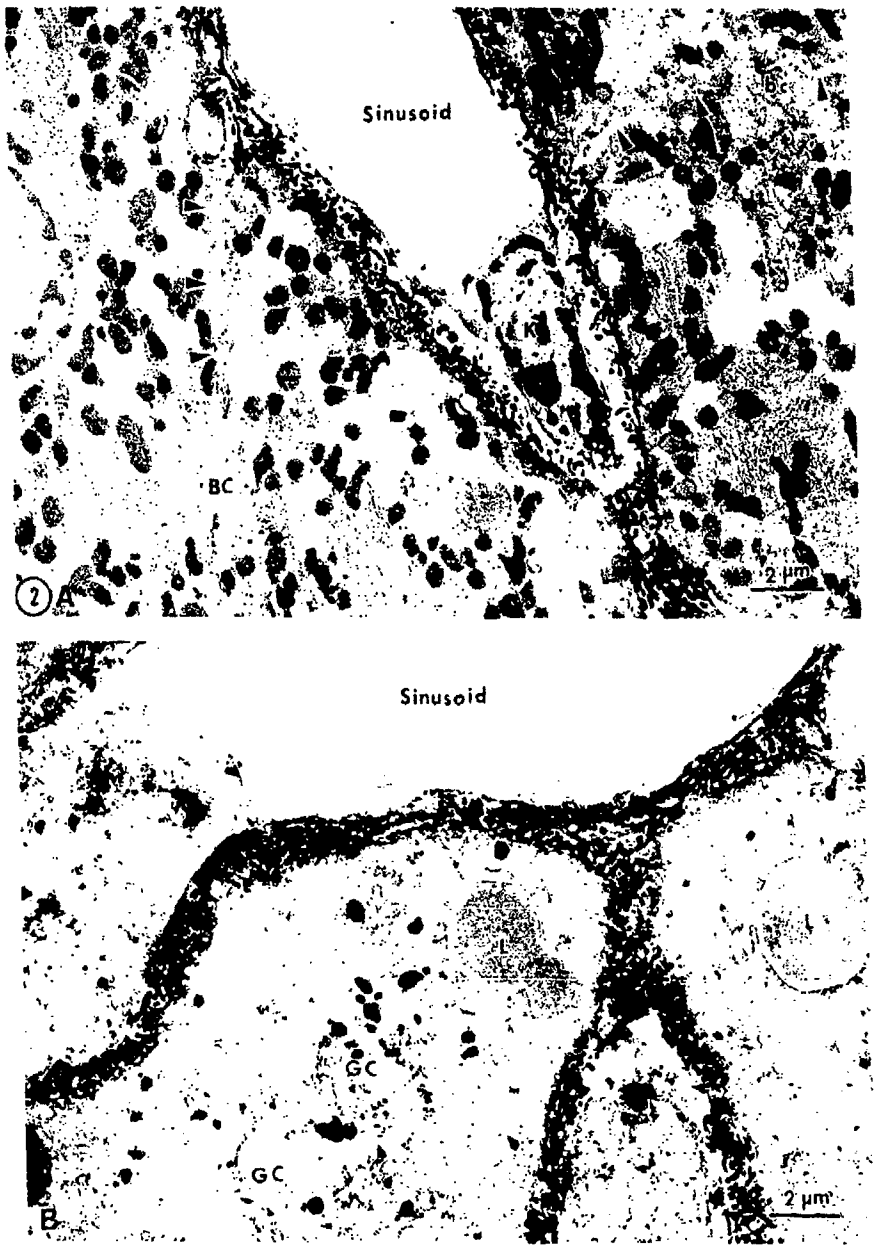


Figure 2. Immunoelectron microscopic localization of fibronectin in hepatocytes. Electron-dense HRP reaction product is localized on the basal (perisinusoidal) surfaces. (A) Hepatocytes of control rats. The basal surfaces of the cells are moderately stained, whereas the apical (pericanalicular), lateral surfaces, Kupffer (K) cells, and endothelial cells are not stained. Arrowheads indicate the lateral surfaces of the hepatocytes. BC, bile canaliculus. (B) The basal surfaces of the hepatocytes from animals suffering endotoxemia or septic shock are more intensely stained. L, lipid droplet; GC, Golgi complex. Original magnifications × 6320.

pH 7.2, containing 0.15% cytochrome c (Sigma) at room temperature for 15–20 min (Angermuller and Fahimi, 1984).

Catalase. The procedure reported by Novikoff and Goldfischer (1969) was used to localize catalase in the peroxisomes of hepatocytes.

Adenosine Triphosphatase (ATPase). Vibratome sections were incubated in a medium containing the sodium salt of adenosine triphosphate (Sigma), magnesium sulfate, and lead nitrate at 37° C for 35–40 min (Wachstein and Meisel, 1957).

Injection with Horseradish Peroxidase (HRP) Tracer. Three endotoxemic and three control rats were given 10 mg/ml HRP (Sigma) in saline by intravenous injection via a saphenous vein. Animals were sacrificed by prolonged inhalation of halothane 15 min after injection. Middle lobes of the livers were removed and immediately fixed in 2% glutaraldehyde–1% paraformaldehyde fixative for 30–45 min. Vibratome sections (70–400 μm) were incubated in a DAB medium (Graham and Karnovsky, 1966) at room temperature for 35 min and post-fixed overnight in 2% osmium tetroxide without or with 1.5% potassium ferrocyanide for 2 hr at 4°C.

Electron Microscopy. All samples except those used for glycogen and calcium localization and tracer study were post-fixed in 2% osmium tetroxide at 4°C for 2 hr. After washing in 0.1 M sodium cacodylate buffer, samples were dehydrated in a series of graded ethanols and embedded in Epon (Polybed; Polysciences, Warrington, PA). Ultra-thin sections were prepared with a diamond knife and lightly stained with lead citrate. Sections were examined in a JEOL 100 CX transmission electron microscope. One-μm sections were also prepared from samples processed for fibronectin localization, using

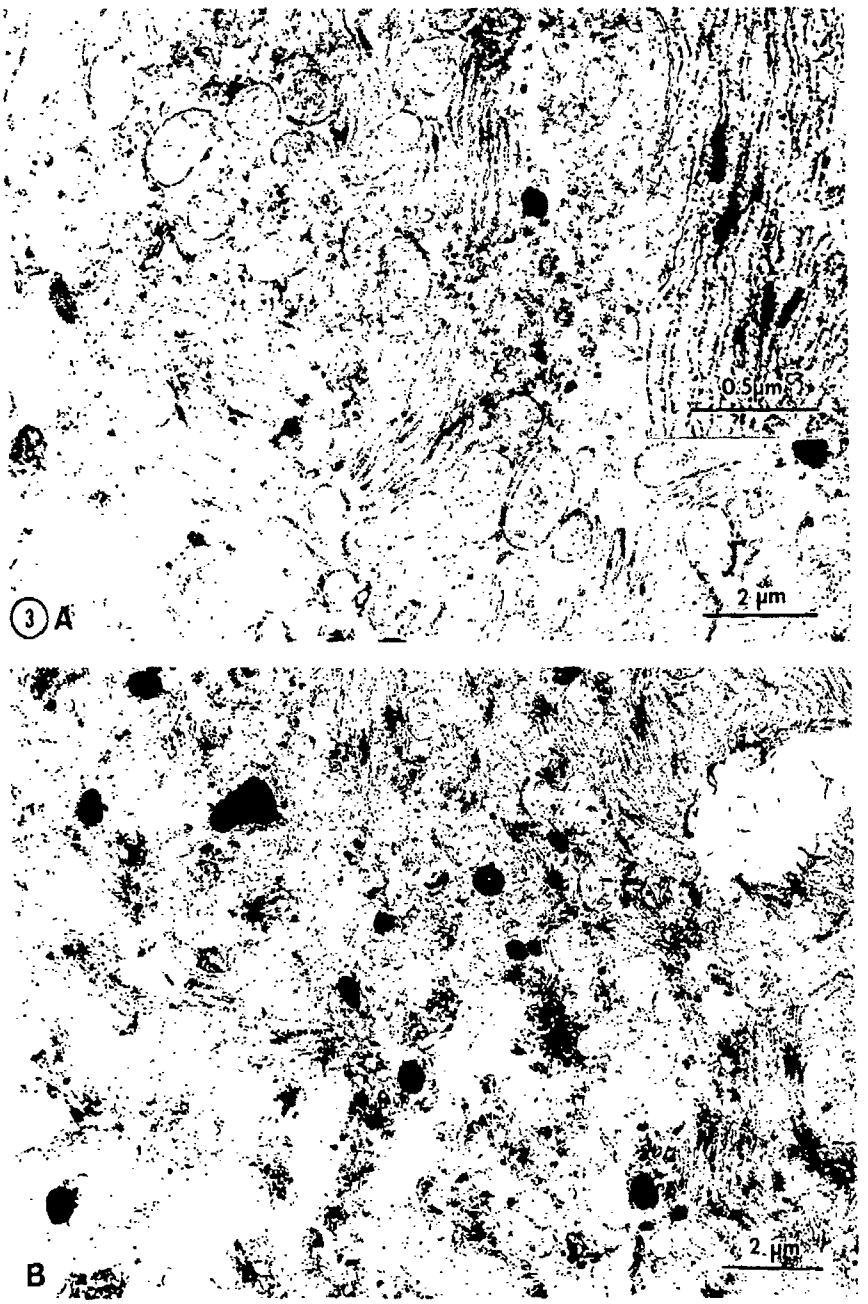


Figure 3. Intracellular distribution of fibronectin in rat hepatocytes. Only portions of the rough endoplasmic reticulum (RER) are stained for fibronectin. (**A**) Hepatocytes of control rats. Only a few RER cisternae exhibit staining of fibronectin. Inset shows a higher magnification of RER with fibronectin staining. (**B**) Hepatocytes of rats with endotoxemia or septic shock. Many RER cisternae are stained. Dark bodies in the cytoplasm are peroxisomes. Original magnifications: **A**, **B**, × 10,000; inset × 44,000.

glass knives, and were photographed with a Zeiss photomicroscope without counterstaining.

Statistical Evaluation. The numbers of peroxisomes in hepatocytes were quantitated by counting the numbers of this organelle per cell section through a nuclear plane, and were expressed as means ± SD. Data were analyzed using Student's *t*-test. A total of 1500 mitochondria per sample, with or without cytochrome *c* oxidase reactivity, were counted. Data were expressed as percent of mitochondria displaying enzyme activity.

Results

Light microscopic localization of tissue fibronectin in the liver was indicated by a brown stain or, with electron microscopy, by an electron-dense deposit of HRP reaction product. Results from light microscopic observations showed that the hepatic sinusoids of the LPS-treated or septic rats (Figure 1A) were more intensely stained for fibronectin than those in the controls (Figure 1B). Immunoelectron microscopic localization showed results corresponding to the light microscopic observations and revealed that only the microvillous basal (perisinusoidal) surfaces of the hepatocytes were stained, whereas the apical (pericanalicular) and lateral surfaces of the cells were not stained (Figure 2A). Kupffer cells and endothelial cells were not stained (Figure 2A). The perisinusoidal surfaces of hepatocytes in the LPS-treated or septic rats were more strongly

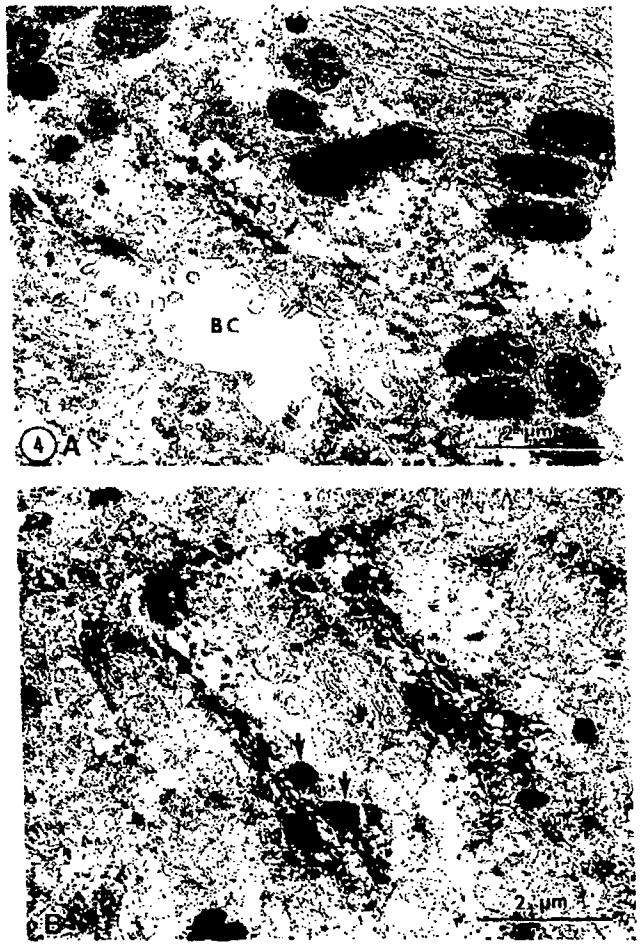


Figure 4. Localization of fibronectin in the Golgi complex of hepatocytes. **(A)** In control rats, only a small portion of the Golgi saccules (arrows) is stained for fibronectin. **(B)** Golgi saccules and vesicles, and secretory granules (thin arrows), are intensely stained in rats with endotoxemia or septic shock. Thick arrows indicate peroxisomes. Original magnifications × 12,450.

stained as compared to controls (Figure 2B). Fibronectin was also localized in the cisternae of RER (Figure 3), Golgi saccules and vesicles (Figure 4), and in small cytoplasmic vesicles of hepatocytes. In the RER, only portions of the cisternae were stained (Figure 3A). Many RER cisternae in the hepatocytes of LPS-treated or septic rats showed fibronectin staining (Figure 3B), in contrast to the relative paucity of staining in RER of normal hepatocytes (Figure 3A). Similarly, the Golgi complex of the hepatocytes from LPS-treated or septic rats exhibited more intense staining for fibronectin than that of the controls (Figure 4).

Calcium ions (Ca^{2+}) were precipitated as an electron-dense calcium pyroantimonate deposit on the outer surfaces of RER and in the mitochondrial matrix and nuclear heterochromatin of rat hepatocytes of both control (Figure 5) and LPS-treated or septic rats (Figure 5). No calcium pyroantimonate precipitate was found in the hepatocytes of liver samples pre-treated with EGTA. More calcium precipitate was observed along the outer surfaces of RER and in the mitochondrial matrix of hepatocytes 30 min post LPS injection (Figure 6A) as compared to those of the controls. The precipitate was more evident and dense along the RER of hepatocytes of endotoxemic rats (Figure 6B) and in the cytoplasm and mitochondria of septic animals (Figure 6C).

Hepatic catalase was localized in spherical membrane-bound peroxisomes (Figure 7A). The number of peroxisomes per cell section (circumference) through the nuclear plane of the hepatocytes of LPS-treated or septic rats was significantly decreased ($p < 0.01$). The shape of the peroxisomes was also changed (Figure 7B). In LPS-treated rats, 17.38 ± 2.5 peroxisomes per hepatocyte section were counted, as compared to 34.55 ± 3.1 peroxisomes in the control hepatocytes. In sham-control and septic rats, 34.71 ± 9.22 and 22.58 ± 2.87 peroxisomes per cell section were found, respectively (Table 1).

Abundant glycogen was normally observed in the hepatocytes of control rats, whereas glycogen was greatly diminished in the LPS-treated rats or totally depleted in the septic rats (Figure 8). The quantity of hepatocellular glycogen exhibited a decreasing magnitude from the centrilobular to the peripheral lobular regions. This was expressed as a decrease in intracellular PAS-positive material which was eliminated with diastase digestion. G-6-Pase was localized in the endoplasmic reticulum, Golgi complex, and nuclear envelope of hepatocytes of both the control and the LPS-treated or septic rats (Figure 9). However, more RER cisternae resulting from glycogen diminution were stained, and the staining seemed more intense in the hepatocytes of the latter than in those of the controls (Figure 9).

Cytochrome *c* oxidase was localized in the spaces between the outer and inner membranes and in the intracristal spaces of mitochondria. In normal rats, more than 90% of the mitochondria in hepatocytes showed positive reactivity of cytochrome *c* oxidase (Figure 10A), whereas in the LPS-treated or septic rats a significant number of mitochondria displayed negative reactivity of the enzyme (Figure 10B). Up to 44 ± 23% and 47 ± 18% of the total mitochondria showed deficiency of cytochrome *c* oxidase in the hepatocytes of LPS-treated rats and septic rats, respectively. Frequently, a few mitochondria in the hepatocytes of LPS-treated or septic rats showed weak enzyme staining only in the same intracristal spaces (Figure 9B).

In control rats, the reaction product of Mg^{2+}-ATPase was localized in the basal, apical, and lateral plasma membranes of the hepatocytes (Figure 11A). The apical (pericanalicular) surfaces were most intensely stained, in both the control and the LPS-treated rats, as previously reported by Meier et al. (1984). In rats treated with LPS, the basal and lateral surfaces of the hepatocytes were weakly stained or totally devoid of the enzyme reaction product (Figure 11B).

The pinocytic activity of hepatocytes was enhanced after treatment with LPS, as indicated by more incorporation of the intravenously injected HRP. Many pinocytic vesicles and large vacuoles containing the reaction product of HRP were observed in the hepatocytes of rats treated with LPS. The tight junctions surrounding the bile canaliculi were not altered by LPS treatment, as demonstrated by occlusion of HRP in the intercellular spaces surrounding bile canaliculi.

The cytochemical alterations in the hepatocytes of normal, LPS-treated, and septic rats are summarized in Table 1.

Figure 5. Calcium localization in the hepatocytes of control rats. Fine calcium pyroantimonate precipitate is distributed on the outer surfaces of RER and in the mitochondrial matrix. Original magnification × 62,990.

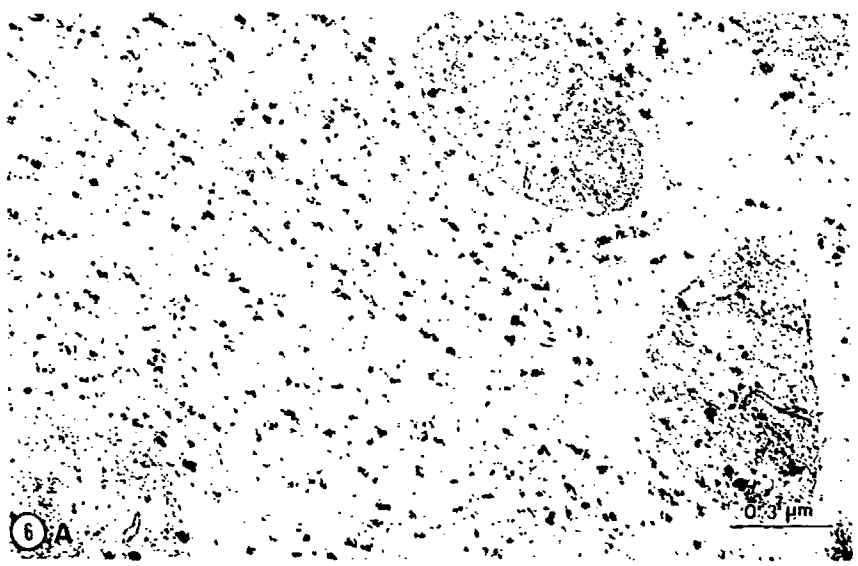
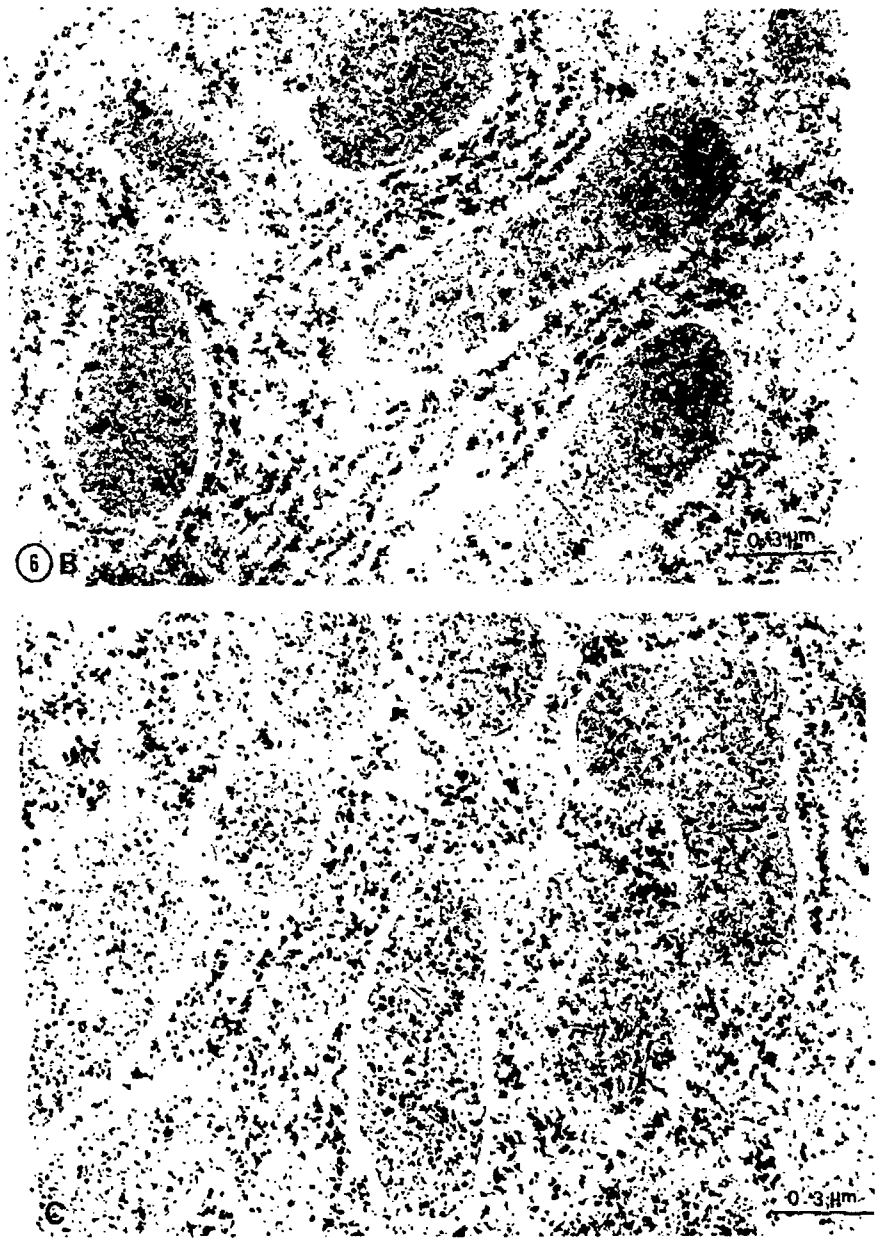


Figure 6. Calcium localization in the hepatocytes of LPS-treated or septic rats. (A) More coarse calcium pyroantimonate precipitate is deposited along the outer surfaces of RER 30 min after LPS injection. (B) Calcium precipitate is significantly increased in the RER and mitochondria 24 hr post injection. (C) Dense calcium precipitate is observed in the cytoplasm and mitochondrial matrix of hepatocytes of septic animals. Original magnifications × 62,990.

Discussion

The pathophysiological differences and similarities between sepsis and endotoxemia are well understood. Animals with sepsis induced by cecal ligation and puncture show positive cultures for various enteric microorganisms, hypodynamic circulation, hypoinsulinemia, hypoglycemia, high serum lactate levels, and decreased blood flow to the organs (Wichterman et al., 1980). Endotoxemia is characterized by hypotension associated with peripheral resistance, low cardiac output (Swan and Jacobson, 1967), a transient hyperglycemia, and terminal hypoglycemia (Hinshaw, 1976). O'Donnell et al. (1957) have shown that endotoxin inhibits hepatic gluconeogenesis in pigs, whereas sepsis produced by cecal ligation and puncture stimulates hepatic conversion of lactate and alanine to glucose. In the present studies, we have observed similar cytochemical alterations in the hepatocytes of rats suffering from endotoxemia or from cecal ligation- and puncture-induced sepsis. These changes included glycogen diminution or depletion, increased tissue fibronectin, decrease of peroxisomes, and an increase of cytochrome *c* oxidase-deficient mitochondria. Deposition of fibrin in sinusoids, depletion of glycogen, vacuolation of mitochondria, and dilation of endoplasmic reticulum are known to be important cellular signs of endotoxemic and shock symptoms in animals (De Palma et al., 1967, 1970; Rangel et al., 1970; Schumer et al., 1970; Moss et al., 1969; McKay et al., 1966; Hift and Strawitz, 1961).

Fibronectin plays an important role in RES clearance of particulate matter such as fibrin, collagen, and actin (Gold and Pearlstein, 1981; Keski-Oja et al., 1980), thus preventing excessive localization of these materials in highly vascular organs, such as lung



and kidney (Saba and Jaffe, 1980). Recently, Richards and Saba (1985) reported that intravenous administration of *E. coli* LPS to rats significantly elevated plasma fibronectin levels. The enhanced fibronectin was found to be directly associated with the phagocytic activity of Kupffer cells. This correlation leads some authors to believe that depletion of fibronectin is directly related to increased severity of sepsis (Richards and Saba, 1985; Velky et al., 1984; Kaplan, 1981). On the contrary, Grossman et al. (1983) have reported that there is no direct relationship between fibronectin and sepsis.

In the present studies, the intense staining of fibronectin in the basal (perisinusoidal) surfaces of hepatocytes in the endotoxemic and septic rats suggests that two events may have occurred in the hepatocytes during the process of the illness: (a) enhanced incorporation of plasma fibronectin into the basal plasma membrane of hepatocytes; and (b) active synthesis and secretion of fibronectin. In fact, increased plasma fibronectin levels after LPS treatment (Richards and Saba, 1985) and incorporation of plasma fibronectin into tissues (Deno et al., 1983) have both been reported. In septic or endotoxemic rats, the presence of many stained RER and Golgi complexes in the hepatocytes indicates active synthesis of fibronectin. This finding agrees with reports that increased synthesis of fibronectin was found in animals with cecal ligation-induced sepsis (Velky et al., 1983, 1984) and that plasma fibronectin was elevated in LPS-treated rats (Richards and Saba, 1985).

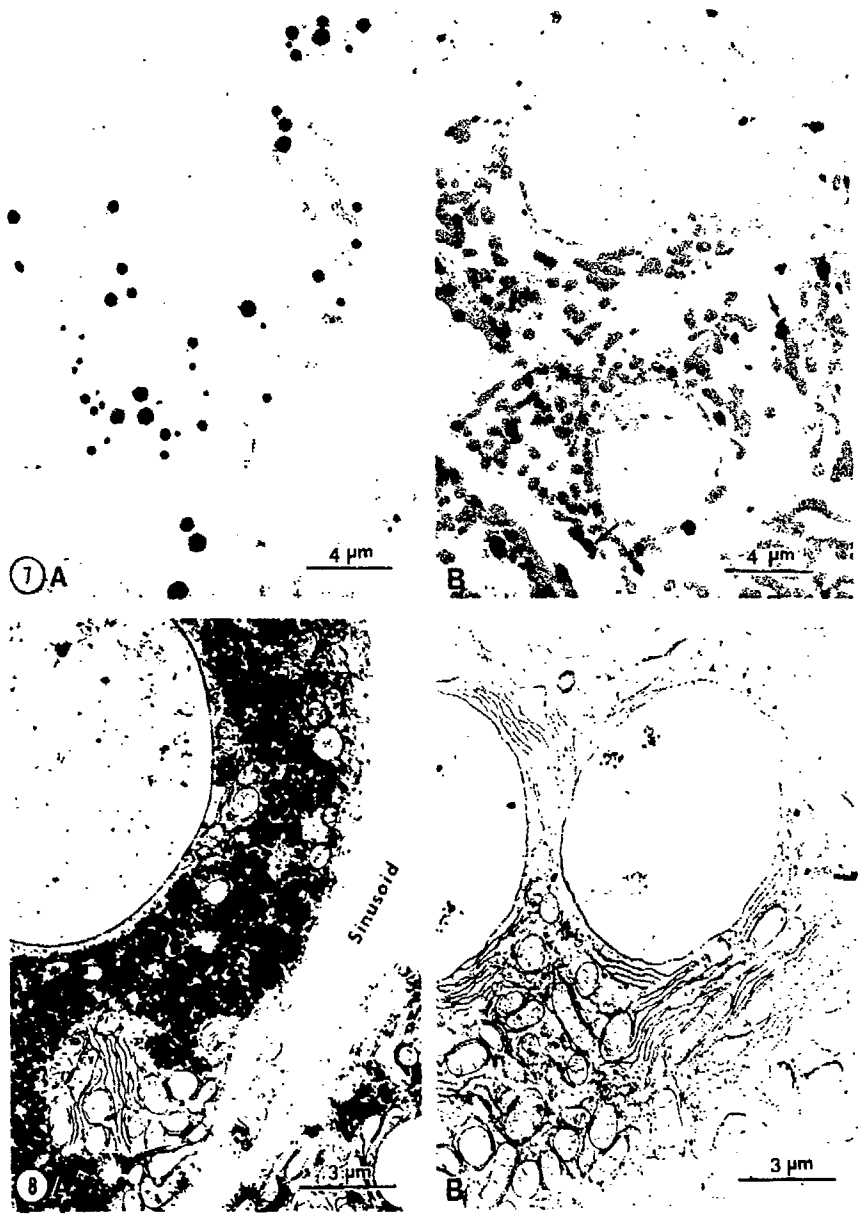


Figure 7. Localization of catalase in hepatic peroxisomes. Many peroxisomes are observed in normal hepatocytes (**A**), whereas only a few peroxisomes (arrows) are seen in the hepatocytes of rats with endotoxemia or septic shock (**B**). Original magnifications × 4320.

Figure 8. Localization of glycogen in hepatocytes using OsFeCN post-fixation method. (**A**) Abundant glycogen is present in the hepatocytes of normal control rats. (**B**) Glycogen is greatly diminished or totally depleted in the hepatocytes of septic rats. Original magnifications × 6400.

Moreover, others have localized fibronectin to the RER cisternae and Golgi complex of fibroblasts (Yamada et al., 1980), smooth muscle cells (Chemnitz and Christensen, 1983), and hepatocytes (Clement et al., 1985). It has been reported that fibronectin binds to LPS and facilitates phagocytosis (Porvaznik et al., 1982). Thus, the increases in plasma fibronectin (Richards and Saba, 1985) and tissue fibronectin in hepatocytes of endotoxemic or septic rats (present studies) may enhance removal of LPS from blood by the RES system and hepatocytes.

Free calcium in the cell regulates muscle contraction, metabolic processes, hormone and transmitter secretion, and membrane transport and permeability. Recent studies have indicated that endoplasmic reticulum is the main organelle regulating cytoplasmic Ca^{2+} concentration (Somlyo et al., 1985; Somlyo, 1984). Cytochemical localization of calcium in the present studies also showed that Ca^{2+} was predominantly precipitated on the outer surfaces of the RER and sparsely in the mitochondrial matrix of hepatocytes in normal animals. In the hepatocytes of LPS-treated or septic rats, precipitation of Ca^{2+} at the same sites was prominently increased. Studies of the effect of endotoxin on cytoplasmic Ca^{2+} concentration have not been conclusive. Kilpatrick Smith and Erecinska (1983) indicated that *E. coli* LPS decreases the intracellular Ca^{2+} concentration in mouse neuroblastoma cells. Nicholas et al. (1972) have reported that endotoxemia also causes severe inhibition of liver mitochondrial calcium uptake function. Moreover, a recent report indicates that endotoxin induces a decrease in calcium content in

Table 1. *Cytochemical alterations in hepatocytes of LPS-treated or septic rats as compared to control animals*[a]

	Control	LPS-treated	Septic
Fibronectin			
Cell surface	+	+ +	+ +
Intracellular	+	+ +	+ +
Ca^{++}			
RER	+	+ +	+ +
Mitochondria	+	+ +	+ +
Glycogen	+ +	+ / + −	−
G-6-Pase	+	+ +	+ +
Peroxisomes[b]	34.55 ± 3.1		
	34.71 ± 9.22	17.38 ± 2.59	22.58 ± 2.87
Cytochrome *c* oxidase[c]	93%	56%	53%
ATPase (plasma membrane)			
Basal	+	+ −	ND
Lateral	+	+ −	ND
Apical	+ +	+ +	ND

[a] + +, high; +, moderate; + −, weak; −, negative; ND, not done.
[b] The number of peroxisomes per cell section of hepatocytes in rats with endotoxemia or sepsis was significantly decreased (endotoxemic, $p = 0.001$; septic, $p = 0.01$).
[c] Number of mitochondria with cytochrome *c* oxidase reactivity.

the microsomal fraction of liver (Deaciuc and Spitzer, 1987). However, other reports have indicated that bacterial endotoxin potentiates the influx of Ca^{2+} in various cell types (Hulsmann et al., 1981; Nelson and Spitzer, 1981; Connor et al., 1973) and enhances energy-independent Ca^{2+} binding (Nicholas et al. 1972).

In the present studies, the increase of calcium precipitate in the RER and mitochondria of hepatocytes from LPS-treated or septic animals indicates an increase of Ca^{2+} uptake by hepatocytes. This result is in agreement with the reports on calcium overload in cardiocytes (Hulsmann et al., 1981) and hepatocytes (Sayeed, 1986) after endotoxin treatment. In rat hepatocytes, cytochemical localization of Ca^{2+} indicates that Ca^{2+} is located predominantly in: (a) membrane associated Ca^{2+} in the endoplasmic reticulum which may be bound to membrane phospholipids (phosphatidylserine and polyphosphoinositides) (Lullman and Peters, 1977; Buckley and Hawthorn, 1972); and (b) non-membrane-associated Ca^{2+} in the mitochondrial matrix. The latter may leak from mitochondria subsequent to damage to mitochondrial membrane after prolonged treatment with LPS (Nicholas et al., 1972). The accumulation of calcium in the mitochondria may result in swelling and vacuolation of mitochondria (Constantinides, 1984). The calcium overload in hepatocytes may contribute to metabolic alterations of carbohydrates related to septic shock (Sayeed, 1986).

McCallum (1981) has shown that LPS impairs glycogenesis and gluconeogenesis. The impaired metabolism is correlated with reduced activities of glycogen synthase *a*, phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, and fructose 1,6-diphosphatase. In the present studies, however, we found that augmented increase of G-6-Pase activity, as demonstrated by increased staining of the enzyme in the RER, showed a positive correlation with glycogen diminution or depletion in hepatocytes of LPS-treated or septic rats. Enhancement of G-6-Pase activity would increase glycogenolysis (Freeland and Harper, 1966). It has been reported that transient hyperglycemia due to glycogenolysis is observed in endotoxemic animals (Hinshaw, 1976), but later a profound and terminal hypoglycemia ensues as glycogen stores are depleted and gluconeogenesis is inhibited (Filkins and Cornell, 1974).

Hepatic peroxisomes are involved in beta-oxidation of lipids (Lazarow, 1978; Lazarow and De Duve, 1976). Deficiency of peroxisomes in humans has been reported to be fatal in infants with Zellweger's cerebrohepatorenal syndrome (Goldfischer, 1982). The biogenesis of peroxisomes and synthesis of catalase in hepatocytes are dependent on polysomes and endoplasmic reticulum (Lazarow et al., 1982; Masters, 1982). Geerts and Roels (1982) reported that diminished catalase availability results in reductions in both peroxisome volume and number. We observed morphological alterations and a significant reduction of peroxisomes in the hepatocytes of septic or LPS-treated rats. This finding indicates that LPS may cause peroxisome degradation. LPS has recently been reported to activate phospholipase activity and to impair arachidonic acid activation (deTurco and Spitzer, 1987). This may also contribute to the reduced number of peroxisomes in hepatocytes from LPS-treated or septic rats.

Cytochrome *c* oxidase is a mitochondrial enzyme responsible for consumption of virtually all the molecular oxygen used by eukaryotes during aerobic metabolism. We found that this enzyme was present in all mitochondria in the hepatocytes of normal rats, but was deficient in a significant number of mitochondria in the hepatocytes of LPS-treated and septic rats. Moreover, LPS has been reported to depress the activities of several metabolic enzymes (McGivney and Bradley, 1979) and ATPase (Mela et al., 1971), and to decrease oxygen consumption and phosphorylation (Mager and Theodor, 1957) by liver mitochondria. The decrease of ATPase activity may be related to accumulation of intracellular calcium in the hepatocytes of LPS-treated rats.

In summary, significant cytochemical alterations, including increases in fibronectin and intracellular calcium, increased G-6-Pase activity, a reduction of catalase-containing peroxisomes, and depletion of cytochrome *c* oxidase in mitochondria, were observed in hepatocytes of endotoxemic and septic rats. The overall results suggest that: (a) LPS stimulates hepatic synthesis of fibronectin, which may result in the elevation of plasma fibronectin; (b) LPS stimulates calcium uptake by hepatocytes; (c) LPS stimulates G-6-Pase activity, which may be directly involved in the glycogenolysis characteristic of sepsis; and (d) LPS reduces peroxisome number and depletes mitochondrial cytochrome *c* oxidase, and may have a role in hepatic injury and malfunction.

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Literature Cited

Angermuller S, Fahimi HD (1983): Selective staining of cell organelles in

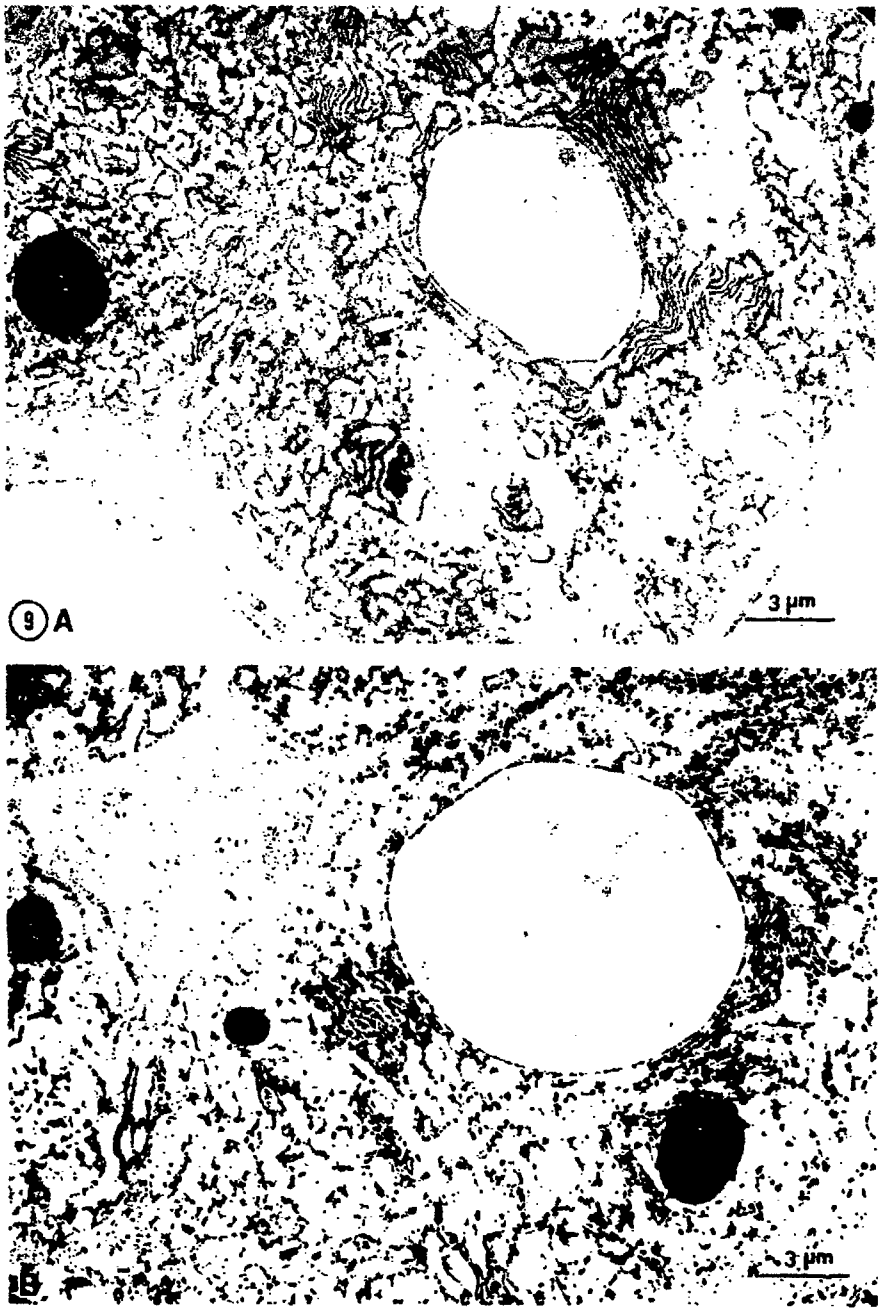

Figure 9. G-6-Pase activity in rat hepatocytes. Enzyme reaction product is localized in the cisternae of RER and the nuclear envelope. Hepatocytes of control rats (**A**) are less intensely stained for the enzyme than those of rats with endotoxemia or septic shock (**B**). L, lipid droplet. Original magnifications × 5280.

rat liver with 3,3'-diaminobenzidine. J Histochem Cytochem 31:230

Appleton J, Morris DC (1979): The use of the potassium pyroantimonate–osmium method as a means of identifying and localizing calcium at the ultrastructural level in the cells of calcifying systems. J Histochem Cytochem 27:676

Berry LJ (1977): Bacterial toxins. CRC Crit Rev Toxicol 5:239

Berry LJ, Rippe DF (1973): Effect of endotoxin on induced liver enzymes. J Infect Dis 128:118

Boler RK, Bibighaus AJ (1967): Ultrastructural alterations of dog livers during endotoxin shock. Lab Invest 17:537

Bradley SG (1979): Cellular and molecular mechanisms of action of bacterial endotoxins. Annu Rev Microbiol 33:67

Buckley JT, Hawthorne JN (1972): Erythrocyte membrane polyphosphoinositide metabolism and the regulation of calcium binding. J Biol Chem 247:7218

Chemnitz J, Christensen B (1983): Intracellular and extracellular localization of fibronectin in primary culture of aortic smooth muscle cells using immunoperoxidase cytochemistry in electron microscopy. Eur J Cell Biol 30:200

Clement B, Rissel M, Peynol S, Mazurier Y, Grimaud J-A, Guillouzo A (1985): A procedure for light and electron microscopic intracellular immunolocalization of collagen and fibronectin in rat liver. J Histochem

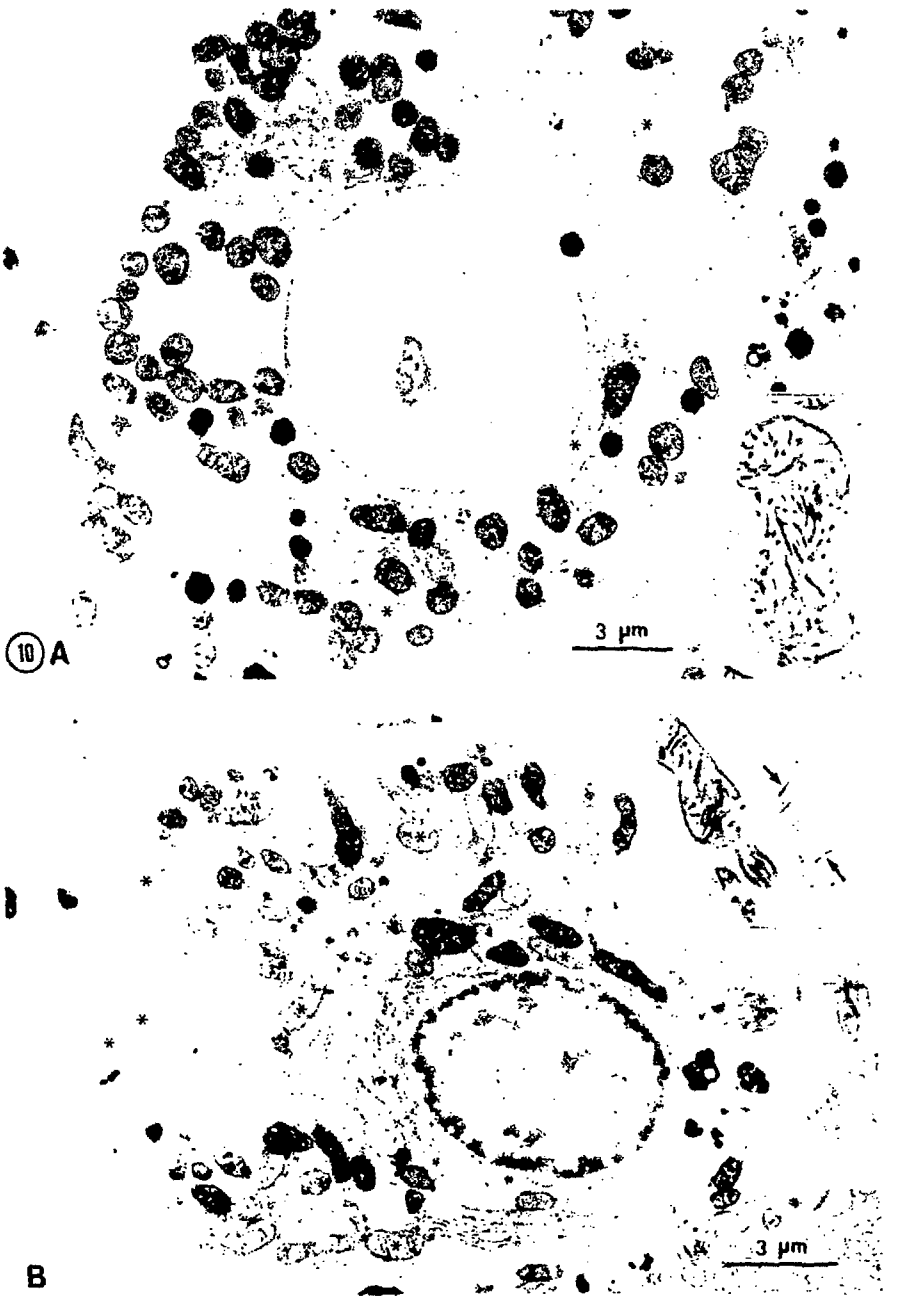

Figure 10. Cytochrome c oxidase in mitochondria of hepatocytes. The enzyme is localized in spaces between outer and inner membranes and in intracristal spaces of mitochondria. (**A**) Hepatocytes of normal control rats. Inset is a higher magnification of a mitochondrion showing distribution of the enzyme. Asterisks indicate a small number of mitochondria showing negative reactivity to the enzyme. (**B**) Hepatocytes of LPS-treated or septic rats. A large number of mitochondria show depletion of the enzyme (asterisks). Inset is a higher magnification of a mitochondrion showing weak staining of the enzyme in the intracristal spaces (arrows). Original magnifications × 6000; insets × 23,380.

Cytochem 33:407

Connor J, Finc J, Kusano K, McCrea MJ, Parnas I, Prosser CL (1975): Potentiation by endotoxin of responses associated with increases in calcium conductance. Proc Natl Acad Sci USA 70:3301

Constantinides P (1984): Ultrastructural pathobiology. New York, Elsevier

Cook JA, Roger TS, Wise WC, Halushka PV (1985): Agents which modulate reticuloendothelial function and affect endotoxin sensitivity. In Berry LJ, ed: Cellular biology of endotoxin. New York, Elsevier, 303

Deactue IV, Spitzer JA (1987): Calcium content in liver and its intracellular distribution during endotoxicosis and sepsis in rats. Fed Proc 46:1432

Demo DC, Saba TM, Lewis EP (1983): Kinetics of endogenously labelled fibronectin incorporation into tissues. Am J Physiol 245:R564

DePalma RG, Coil J, Davis JH, Holden WD (1967): Cellular and ultrastructural changes in endotoxemia: A light and electron microscopic study. Surgery 62:505

DePalma RG, Levey S, Holden WD (1970): Ultrastructure and oxidative phosphorylation of liver mitochondria in experimental hemorrhagic shock. J Trauma 10:122

de Turco ER, Spitzer JA (1987): Glycerolipid content and metabolism in hepatocytes of endotoxin-infused rats. Fed Proc 46:1431

Egawa K, Kasai N (1979): Endotoxic glycolipid as a potent depressor of the hepatic drug-metabolizing enzyme systems in mice metabolism. Microbiol Immunol 23:87

Fawcett DW, Dome M (1974): A glycogen rich segment of the tubuli recti

Figure 11. Localization of ATPase in rat liver. Reaction product of the enzyme is localized on the basal (perisinusoidal), apical (pericanalicular), and lateral surfaces of hepatocytes. Bile canaliculi (BC) are most intensely stained. Basal and lateral surfaces of the hepatocytes in normal rats (**A**) are more intensely stained than those of LPS-treated animals (**B**). Original magnifications × 8660.

and proximal portion of the rete testis in the guinea pig. J Reprod Fertil 39:401

Filkins JP, Cornell RP (1974): Depression of the hepatic gluconeogenesis and the hypoglycemia of endotoxin shock. Am J Physiol 227:778

Fine J, Rotenberg S, Schweinburg FB (1959): The role of the reticuloendothelial system in hemorrhagic shock. J Exp Med 110:547

Freeland RA, Harper AE (1956): Initiation of glucose-6-phosphatase adaptation in the rat. J Nutr 89:429

Geerts A, Roels F (1982): Cytoplasmic and peroxisomal catalase during synthesis inhibition. Ann NY Acad Sci 386:537

Gold LI, Pearlstein E (1981): Fibronectin-collagen binding and requirements during cellular adhesion. Biochem J 186:551

Goldfischer S (1982): Peroxisomes and human metabolic diseases: the cerebrohepatorenal syndrome (CHRS), cerebrotendinous xanthomatosus, and Schilder's disease (adrenoleukodystrophy). Ann NY Acad Sci 386:536

Graham RC, Karnovsky MJ (1966): The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. J Histochem Cytochem 23:291

Grossman J, Pohlman T, Koerner F, Mosher D (1983): Plasma fibronectin concentration in animal models of sepsis and endotoxemia. J Surg Res 34:145

Gut J-P, Schmitt S, Bingen A, Anton M, Kirn A (1984): Probable role of endogenous endotoxins in hepatocytolysis during murine hepatitis caused by frog virus 3. J Infect Dis 149:621

Hift H, Strawitz JG (1961): Irreversible hemorrhagic shock in dogs: problem of onset of irreversibility. Am J Physiol 200:269

Hinshaw LB (1976): The role of glucose in endotoxin shock. Circ Shock 3:1

Hulsmann WC, Lamers JMJ, Stam H, Breeman J (1981): Calcium overload in endotoxemia. Life Sci 29:1009

Kaplan JE (1981): Plasma fibronectin and resistance to thrombosis during sepsis. Circ Shock 8:225

Keski-Oja, San A, Todaro GJ (1980): Direct association of fibronectin and actin molecules *in vitro*. J Cell Biol 85:527

Kilpatrick-Smith L, Erecinska M (1983): Cellular effects of endotoxin *in vitro*: I. Effect of endotoxin on mitochondrial substrate metabolism and intracellular calcium. Circ Shock 11:85

Lanser ME, Saba TM (1983): Correction of serum opsonic defects after burn and sepsis by opsonic fibronectin administration. Arch Surg 118:338

Lazarow PB (1978): Rat liver peroxisomes catalyze the β oxidation of fatty acids. J Biol Chem 253:1522

Lazarow PB, DeDuve C (1976): A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by colfibrate, a hypolipidemic drug. Proc Natl Acad Sci USA 73:2043

Lazarow PB, Robbi M, Fugiki Y (1982): Biogenesis of peroxisomal proteins *in vivo* and *in vitro*. Ann NY Acad Sci 386:285

Levy E, Shesser RJ, Ruebner BH (1968): Hepatic changes produced by a single dose of endotoxin in the mouse. Am J Pathol 52:477

Lullman H, Peters T (1977): Plasmalemmal calcium in cardiac excitation–contraction coupling. Clin Exp Pharmacol Physiol 4:49

Mager J, Theodor E (1957): Inhibition of mitochondrial respiration and uncoupling oxidative phosphorylation by fractions of the *Shigella paradysenteriae* Type III somatic antigen. Arch Biochem Biophys 67:169

Maier RV, Ulevitch RJ (1981): The response of isolated hepatic rabbit macrophages (H-MØ) to lipopolysaccharide (LPS). Circ Shock 8:165

Masters CJ (1982): On the turnover and latency of peroxisomal catalases. Ann NY Acad Sci 386:301

Mathison JC, Ulevitch RJ (1979): The clearance, tissue distribution, and cellular localization of intravenously injected lipopolysaccharide in rabbits. J Immunol 123:2133

McCafferty MH, Saba TM (1982): Comparative influence of blood-borne nonbacterial particles and *Staphylococcus aureus* on fibronectin, complement, and immunoglobulin. Ann Surg 196:715

McCallum RE (1981): Hepatocyte–Kupffer cell interactions in the inhibition of hepatic gluconeogenesis by bacterial endotoxin. In Majde JA, Person RJ, eds. Pathophysiological effects of endotoxins at the cellular level. New York, Alan R Liss, 99

McGivney A, Bradley SG (1979): Effects of bacterial endotoxin on lysosomal and mitochondrial enzyme activities of established cell cultures. J Reticuloendothel Soc 26:307

McKay DG, Margaretten W, Csavossy I (1966): An electron microscope study of the effects of bacterial endotoxin on the blood–vascular system. Lab Invest 15:1815

McLean IW, Nakane PK (1974): Periodate–lysine–paraformaldehyde fixative: a new fixative for immunoelectron microscopy. J Histochem Cytochem 22:1077

Meier PJ, Sztul ES, Reuben A, Boyer JL (1984): Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. J Cell Biol 98:991

Mela L, Bacalzo IV, Miller LD (1971): Defective oxidative metabolism of rat liver mitochondria in hemorrhagic and endotoxin shock. Am J Physiol 220:571

Mosher DF, Williams EM (1978): Fibronectin concentration is decreased in plasma of severely ill patients with disseminated intravascular coagulation. J Lab Clin Med 91:729

Moss GS, Erve P, Schumer W (1969): Effect of endotoxin on mitochondrial respiration. Surg Forum 20:24

Nelson KM, Spitzer JA (1983): Calcium uptake and efflux in adipocytes after endotoxin administration. Circ Shock 8:224

Nicholas BB, Mela LM, Miller LD (1972): Shock induced alterations of mitochondrial membrane transport: effect of endotoxin and lysosomal enzymes on calcium transport. Ann Surg 176:579

Nicholas BA, Setzer PY, Bainton DF (1984): Glucose-6-phosphatase as cytochemical marker of endoplasmic reticulum in human leukocytes and platelets. J Histochem Cytochem 32:165

Novikoff AB, Goldfischer S (1969): Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. J Histochem Cytochem 17:675

Nowotny A (1969): Molecular aspects of endotoxic reactions. Bacteriol Rev 33:72

O'Donnell T, Clowes GHA, Ryan NT (1957): Experimental endotoxemia: does it stimulate metabolism in septic shock. Surg Forum 26:25

Palmeria C, Fine J (1969): The nature of resistance to shock. Arch Surg 98:679

Porvaznik M, Cohen ME, Bockowski SW, Mueller II EF, Wirthlin MR Jr (1982): Enhancement of cell attachment to a substrate coated with oral bacterial endotoxin by plasma fibronectin. J Periodont Res 17:154

Rangel DM, Byfield JE, Adomin GE, Stevens GH, Lonkalstud EW (1970): Hepatic ultrastructural response to endotoxin shock. Surgery 68:503

Reske SN, Vyska K, Feinendegen LE (1981): In vivo assessment of phagocytic properties of Kuppffer cells. J Nuc Med 22:405

Richards PS, Saba TM (1985): Effect of endotoxin on fibronectin and Kupffer cell activity. Hepatology 5:32

Richards WO, Scovill WA, Shin B (1983): Opsonic fibronectin deficiency in patients with intra-abdominal infection. Surgery 94:210

Saba TM, Cho E (1979): Reticuloendothelial system response to operative trauma as influenced by erythroprecipitate of cold-insoluble globulin therapy. J Reticuloendothel Soc 26:171

Saba TM, Jaffe E (1980): Plasma fibronectin (opsonic glycoprotein): its synthesis by vascular endothelial cells and role in cardiopulmonary integrity after trauma is related to reticuloendothelial function. Am J Med 68:577

Sayeed MM (1986): Alterations in cellular Ca^{2+} regulation in the liver in endotoxic shock. Am J Physiol 250:R884

Schumer W, Gupta TKD, Moss GS, Nyhus LM (1970): Effect of endotoxemia on liver cell mitochondria in man. Ann Surg 171:875

Scovill WA, Amiest SJ, Saba TM (1979): Cardiovascular hemodynamics after opsonic alpha-2-surface binding glycoprotein therapy in injured patients. Surgery 86:284

Somlyo AP (1985): Cellular site of calcium regulation. Nature 309:516

Somlyo AP, Bond M, Somlyo AV (1985): Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly *in vivo*. Nature 314:622

Swan KG, Jacobson ED (1967): Hemodynamics of endotoxin shock in the conscious animals. Surg Gynecol Obstet 125:1041

Tanikawa K, Iwasaki M (1984): Clinical aspects of endotoxemia in liver diseases. In Homma JY, Kanegasaki S, Luderitz O, Shiba T, Westphal O, eds. Bacterial endotoxin; chemical, biological and clinical aspects. Deerfield Beach, Fl. Verlag Chemie, 327

Velky TS, Greenburg AG, Yang JC, Forbes S (1984): Modulators of plasma fibronectin response during sepsis. Surgery 96:190

Velky TS, Yang JC, Greenburg AG (1983): Plasma fibronectin response to sepsis: mobilization or synthesis. J Trauma 23:669

Wachestein M, Meisel E (1957): Histochemistry of hepatic phosphatase at a physiological pH. Am J Clin Pathol 27:13

White RR, Mela L, Bacalzo L, Olofsson K, Miller LD (1973): Hepatic ultrastructure in endotoxemia, hemorrhage, and hypoxia: emphasis on mitochondrial changes. Surgery 73:525

Wichterman KA, Baue AE, Chaudry IH (1980): Sepsis and septic shock—a

review of laboratory models and a proposal. J Surg Res 29:189

Yamada SS, Yamada KM, Willingham MC (1980): Intracellular localization of fibronectin by immunoelectron microscopy. J Histochem Cytochem 28:953

Yoshida M, Egawa K, Kasai N (1982): Effect of endotoxin and its degradation products on hepatic mixed-function oxidase and heme enzyme systems in mice. Toxicol Lett 12:185

Zahlten RN, Rogff TM, Steer CJ (1981): Isolated Kupffer cells, endothelial cells and hepatocytes as investigative tools for liver research:. Fed Proc 40:2460

Zweifach BW (1958): Microcirculatory derangements as a basis for the lethal manifestations of experimental shock. Br J Anaesthesiol 30:466